Research Article
Experimental and Translational Hepatology

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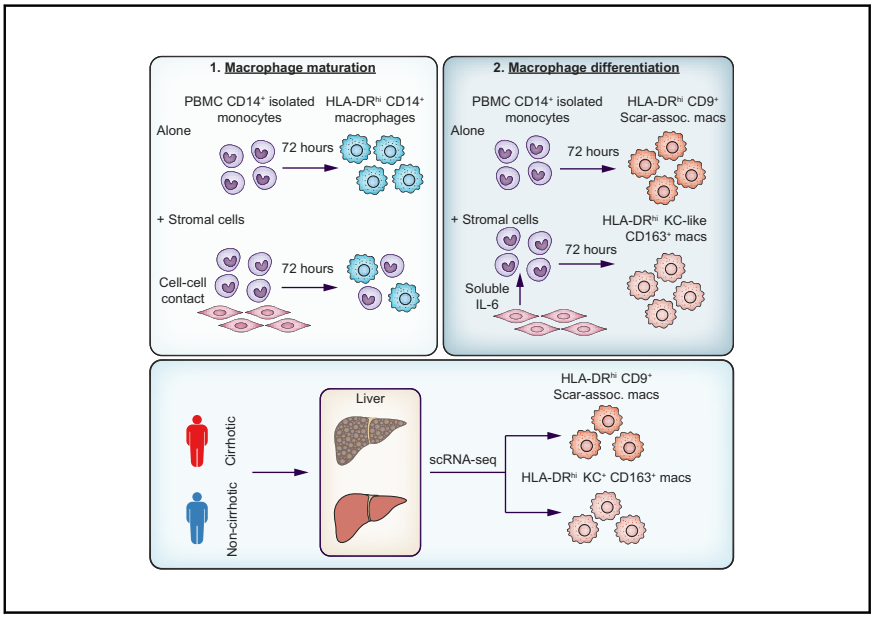
Liver stromal cells restrict macrophage maturation and stromal IL-6 limits the differentiation of cirrhosis-linked macrophages

Graphical abstract



Highlights

- Translated *in vitro* human stromal-myeloid interactions to cells in human cirrhosis.
- Stromal cells limit the transition of $CD14^{+}$ monocytes into $HLA\text{-}DR^{hi}$ macrophages.
- Stromal cell-secreted IL-6 limits the macrophage differentiation into $CD9^{+}$ cirrhotic macrophages.
- Local IL-6 levels are decreased in early-stage human liver disease.

Authors

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Lay summary

The impact of human liver stromal cells on myeloid cell maturation and differentiation in liver disease is incompletely understood. In this study, we present a mechanistic analysis using a primary *in vitro* human liver stromal-myeloid coculture system that is translated to liver disease using single-cell RNA sequencing analysis of cirrhotic and non-cirrhotic human liver tissue. Our work supports a role for stromal cell contact in restricting macrophage maturation and for stromal-derived IL-6 in limiting the differentiation of a cirrhotic macrophage subset.

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Liver stromal cells restrict macrophage maturation and stromal IL-6 limits the differentiation of cirrhosis-linked macrophages

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Background & Aims: Myeloid cells are key regulators of cirrhosis, a major cause of mortality worldwide. Because stromal cells can modulate the functionality of myeloid cells *in vitro*, targeting stromal-myeloid interactions has become an attractive potential therapeutic strategy. We aimed to investigate how human liver stromal cells impact myeloid cell properties and to understand the utility of a stromal-myeloid coculture system to study these interactions in the context of cirrhosis.

Methods: Single-cell RNA-sequencing analyses of non-cirrhotic (n = 7) and cirrhotic (n = 5) human liver tissue were correlated to the bulk RNA-sequencing results of *in vitro* cocultured human $CD14^+$ and primary liver stromal cells. Complimentary mechanistic experiments and flow cytometric analysis were performed on human liver stromal-myeloid coculture systems.

Results: We found that stromal-myeloid coculture reduces the frequency $CD14^+$ cell subsets transcriptionally similar to liver macrophages, showing that stromal cells inhibit the maturation of monocytes into macrophages. Stromal cells also influenced *in vitro* macrophage differentiation by skewing away from cirrhosis-linked $CD9^+$ scar-associated macrophage-like cells and towards $CD163^+$ Kupffer cell-like macrophages. We identify IL-6 production as a mechanism by which stromal cells limit $CD9^+$ macrophage differentiation and find that local IL-6 levels are decreased in early-stage human liver disease compared to healthy liver tissue, suggesting a protective role for local IL-6 in the healthy liver.

Conclusions: Our work reveals an unanticipated role for liver stromal cells in impeding the maturation and altering the differentiation of macrophages and should prompt investigations into the role of local IL-6 production in the pathogenesis of liver disease. These studies provide a framework for investigating macrophage-stromal interactions during cirrhosis.

Lay summary: The impact of human liver stromal cells on myeloid cell maturation and differentiation in liver disease is incompletely understood. In this study, we present a mechanistic analysis using a primary *in vitro* human liver stromal-myeloid coculture system that is translated to liver disease using single-cell RNA sequencing analysis of cirrhotic and non-cirrhotic human liver tissue. Our work supports a role for stromal cell contact in restricting macrophage maturation and for stromal-derived IL-6 in limiting the differentiation of a cirrhotic macrophage subset.

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Introduction

Cirrhosis is the cause of approximately 1 million deaths globally per year, yet effective therapies are lacking.[1] Iterative tissue injury leads to prolonged inflammation, fibrosis, cirrhosis, and potentially hepatocellular carcinoma.[2] Immune responses regulate the balance between healthy tissue repair and the development of liver disease. Cirrhosis arises from various etiologies, including infection and genetics, and is the result of fibrotic tissue replacing healthy liver parenchyma. Cirrhosis is considered a multisystem disease, with immune dysfunction at the core of disease.[3] Recent work acknowledges a role for myeloid cells in cirrhosis-associated immune dysfunction.[4]

Macrophages are crucial to wound healing processes but can also orchestrate disease progression in rodent models of liver injury.[5] The opposing roles of myeloid cells in liver disease may be explained by their heterogeneity.[6–8] Monocytes are recruited to liver tissue during injury, and mature and differentiate into macrophages.[9] Distinct populations of monocyte-derived scar-associated macrophages (SAMs) localize within the fibrotic niche and harbor pro-fibrogenic phenotypes, suggesting the clinical relevance of these cells during cirrhosis.[6] In contrast, tissue-resident Kupffer cells (KC) function to maintain homeostasis by removing bacteria and secreting tolerogenic signals, such as IL-

Keywords: liver; cirrhosis; human; myeloid cells; stromal cells; single-cell RNA-seq; IL-6; macrophage maturation; macrophage differentiation.
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10, in healthy liver tissue.[10] Identification of signals that control macrophage properties may uncover novel therapeutic targets for chronic liver disease.

Macrophages receive differentiation signals in a tissue specific-manner and environmental signals are crucial in directing myeloid cell function.[11] Recent work has shown that hepatic stellate cells and hepatocytes can imprint tolerogenic KC phenotypes in mice.[12] During cirrhosis, tolerogenic properties of macrophages subside and KC subsets are replaced with pro-fibrogenic SAMs.[6] SAMs proliferate locally within scar regions of liver tissue to promote collagen expression by hepatic stellate cells.[6] Stromal-myeloid *in vitro* coculture systems have demonstrated that stromal cells increase the prevalence of monocyte-derived suppressor cells, suggesting stromal cells impact myeloid cell function.[13,14] Additionally, ligand-receptor-pair analyses predict that SAMs can also modulate a fibrotic phenotype in liver stromal cells, suggesting bi-directional stromal-myeloid crosstalk.[6] Collectively, these data suggest that stromal-myeloid interactions may be crucial to the development of liver disease, yet it is unclear how stromal cells affect macrophage maturation and differentiation. Therefore, the development of tools to define mechanisms by which stromal cells control macrophage differentiation may reveal novel molecular pathways responsible for promoting cirrhosis.

We sought to investigate whether stromal-myeloid coculture systems could be leveraged to identify mechanisms that regulate maturation and differentiation of macrophages with clinical relevance to liver disease. We translated bulk RNA-seq, flow cytometric, and mechanistic observations from an *in vitro* human stromal-myeloid coculture system to a scRNA-seq dataset of stromal and myeloid cells isolated *ex vivo* from human non-cirrhotic and cirrhotic tissue.

Materials and methods

Complete methods in supplemental materials.

Study approval

All studies were done under the review and approval of Partners Human Research Committee System Institutional Review Board (IRB). All patients provided anonymous consent.

Results

Single-cell atlas of non-cirrhotic and cirrhotic cells reveals distinct cell populations

We investigated how liver stromal cells regulate myeloid cell phenotypes and the relevance of an *in vitro* stromal-myeloid system to study human cirrhosis using two approaches. First, we characterized the transcriptomes of liver stromal and myeloid cells using single-cell RNA-sequencing (scRNA-seq). Next, we used a primary human stromal-myeloid coculture system to investigate how liver stromal cells impact the maturation and differentiation of $CD14^+$ cells by transcriptional bulk RNA-sequencing (RNA-seq) analysis and cellular phenotyping (Fig. 1A).

Liver cells were isolated from freshly resected human non-cirrhotic and cirrhotic livers (Table S1). Single-cell RNA profiling was performed on 12 liver samples (n = 7 non-cirrhotic, n = 5 cirrhotic). We obtained 38,079 cells; 21,026 from non-cirrhotic and 17,053 from cirrhotic livers. Conos was used to integrate and cluster cells across samples.[15] Joint analysis revealed 14 major clusters spanning immune and non-hematopoietic compartments. Clusters were annotated using known lineage markers (Fig. S1A-E, Table S2). Integration of our data with published scRNA-seq analysis confirmed our cell subset identification (Fig. S1F).[6]

Focused analysis of myeloid lineage signatures revealed 5 major clusters: blood-derived monocytes (Mono1), $CD16^+$ monocytes (Mono2), KCs, SAMs, and myeloid-derived dendritic cells (mDC) (Fig. 1B). Myeloid cell subsets were assigned using key gene markers (Fig. 1B-C, Fig. S1E-F). Mono1 expressed blood-derived monocytic genes (*CD14, LYZ, S100A9, S100A8*). Mono2 expressed $CD16^+$ monocytic genes (*FCGR3A, TIMP1, CD52*). KC cells expressed *C1QC, CD163, CTSB*, *FCGR3A* genes and SAM cells expressed *TREM2*, *CD9, FABP5 and SPP1* genes. mDC cells had low expression of *CD14* and upregulated *CD1C* and *CLEC10A*.

Overall, these data reveal a landscape for single-cell subsets in non-cirrhotic and cirrhotic livers and confirm previously published liver single-cell atlases.

HLA-DR upregulation represents human monocyte to macrophage maturation

We performed bulk RNA-seq on $CD14^+$ cells cultured alone for 0-72 hours to understand how *in vitro* cells align to liver myeloid subsets (Fig. 1A). We generated gene signatures for our scRNA-seq myeloid cell clusters and estimated the relative abundance of these clusters in the cultured $CD14^+$ cells using the diagonally weighted least squares (DWLS) method (Fig. 1D, Fig. S2).[16] As expected, the majority of $CD14^+$ cells at 0 hours transcriptionally aligned with blood-derived monocyte cell subset, Mono1. However, $CD14^+$ cells shifted towards the transcriptional profile of macrophage subsets, KCs and SAMs, after 72 hours. Gene expression analyses of key signature genes in *in vitro* CD14+ cells and *ex vivo* myeloid clusters support the similarities between 0-hour $CD14^+$ cells and monocyte subsets (*S100A12* and *S100A8*) and 72-hour $CD14^+$ cells and macrophage subsets (*CTSB, CD163, CD9, TREM2, FABP5*) (Fig. 1E). These data suggest that $CD14^+$ cells transition from monocytes to macrophages over 72 hours in culture.

Because macrophages are better at presenting antigen than monocytes, we examined HLA-DR expression as a distinguishing marker for human macrophages *vs.* monocytes using scRNA-seq. Analysis of liver tissue revealed upregulation of HLA-DRA expression in macrophage subsets (KCs and SAMs) compared to monocyte subsets (Mono1 and Mono2) (Fig. 1F). To confirm that HLA-DRA upregulation was a reliable marker to define human macrophages across multiple tissue types, we assessed HLA-DRA expression on myeloid cells in human tumors using publicly available scRNA-seq datasets from pancreatic (Fig. S3A-C) and liver (Fig. S3D-F) tumor tissue.[17,18] Increased HLA-DRA expression in macrophage subsets *vs.* monocyte subsets was observed in both tumor types, confirming the applicability of HLA-DRA expression to distinguish human macrophages from monocytes.

Bulk RNA-seq and flow cytometry analysis revealed that HLA-DRA expression was increased in $CD14^+$ cells cultured for 72 hours (Fig. 1G-H). HLA-DRA^{hi} frequencies in $CD14^+$ cells at 0, 24, 48, 72 hours of culture were 1.8%, 12.7%, 76.2%, and 97.1%, respectively. Additionally, we observed that various HLA molecules, such as *HLA-DRB1, HLA-DPB1, HLA-DPA1*, were upregulated in macrophages *vs.* monocytes both in *in vitro* bulk RNA-seq and *ex vivo* scRNA-seq datasets (Fig. S4A-B). We found that

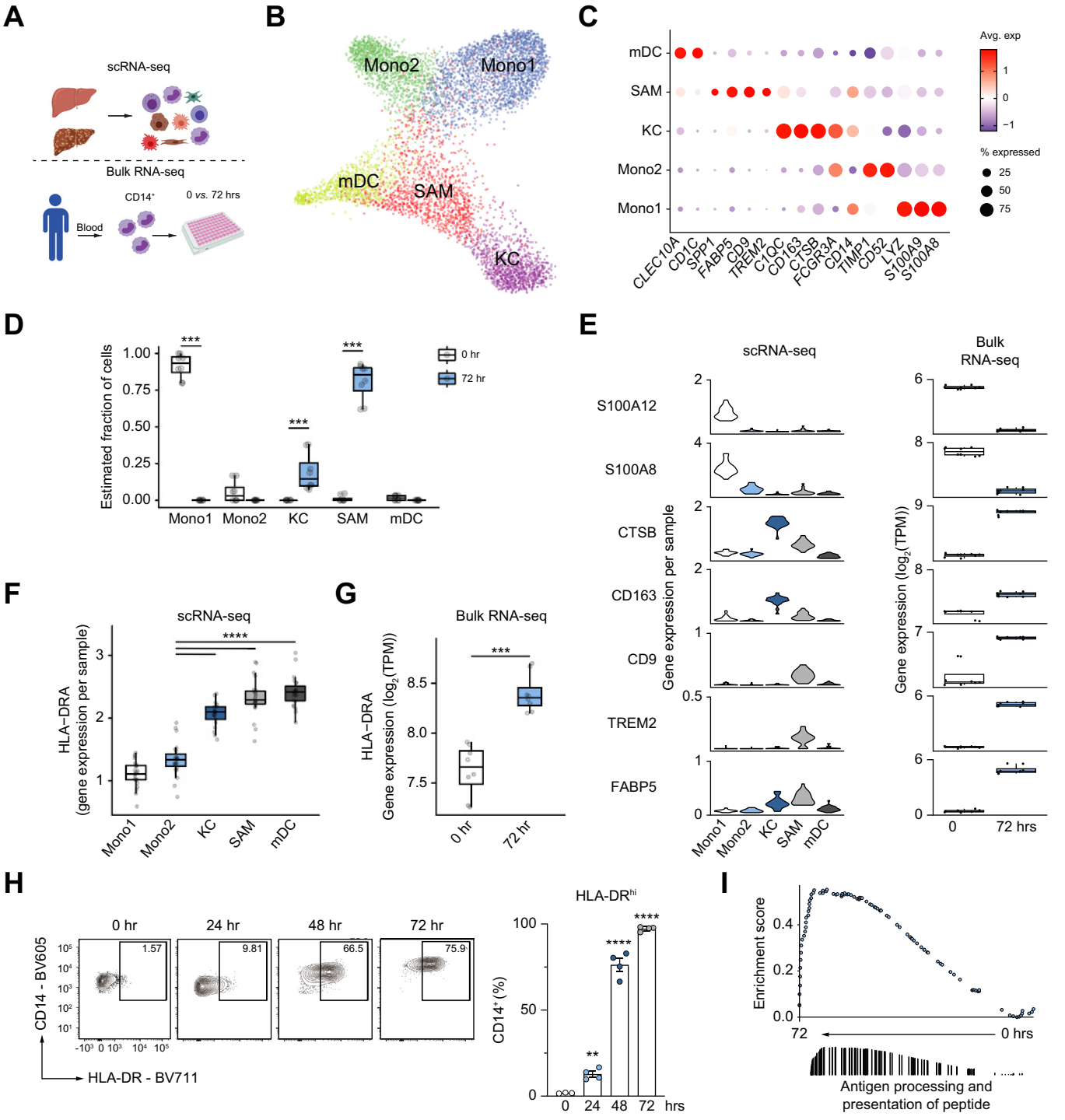

Fig. 1. HLA-DR upregulation on human CD14$^+$ cells indicates macrophage maturation. (A) Experimental schema. (B) UMAP embedding of single-cell myeloid clusters from human livers. (C) Dot plot of gene expression. (D) DWLS boxplot of estimated proportions of scRNA-seq myeloid subsets in bulk RNA-seq dataset of cultured cells. (E) Gene expression from scRNA-seq and bulk RNA-seq datasets. (F-G) HLA-DRA expression in (F) *ex vivo* myeloid clusters and (G) cultured CD14$^+$ cells. (H) Flow cytometric representation and quantification of HLA-DR^{hi} frequency in cultured CD14$^+$ cells. Data are representative of 2 experiments with 2 PBMC donors/experiment. One-way ANOVA Tukey's multiple comparisons. (I) GSEA plot enrichment in bulk RNA-seq dataset of cultured CD14$^+$cells (GO0002504). (D, F) Kruskal-Wallis one-way ANOVA and Dunn's multiple comparisons test; $^{**}p$ <0.005, $^{***}p$ <0.001, $^{****}p$ <0.0001. (G) Wilcoxon rank sum test, $^{***}p$ <0.001. DWLS, diagonally weighted least squares; GSEA, gene set-enrichment analysis; KC, Kupffer cell; mDC, myeloid dendritic cells; PBMC, peripheral blood mononuclear cell; RNA-seq, RNA-sequencing; SAM, scar-associated macrophage; scRNA-seq, single-cell RNA sequencing; UMAP, uniform manifold approximation and projection.

72-hour cultured cells were enriched for genes in the Gene Ontology term "Antigen processing and presentation via MHC class II" (Fig. 1I) and upregulated genes *SPP1, CCL2, KITLG,* suggesting more activated and migratory cell states (Fig. S4C). Ligand-receptor gene pathways such as CSF1/CSF1R, IGF/IGF1R were upregulated and IL15/IL15RA were downregulated in

CD14$^+$ cells cultured for 72 hours providing potential cell-intrinsic binding mechanisms for controlling human macrophage maturation (Fig. S4D). Together, these findings suggest that HLA-DR upregulation identifies human macrophages and indicates that CD14$^+$ monocytes mature into HLA-DR^{hi} macrophages after 72 hours in culture.

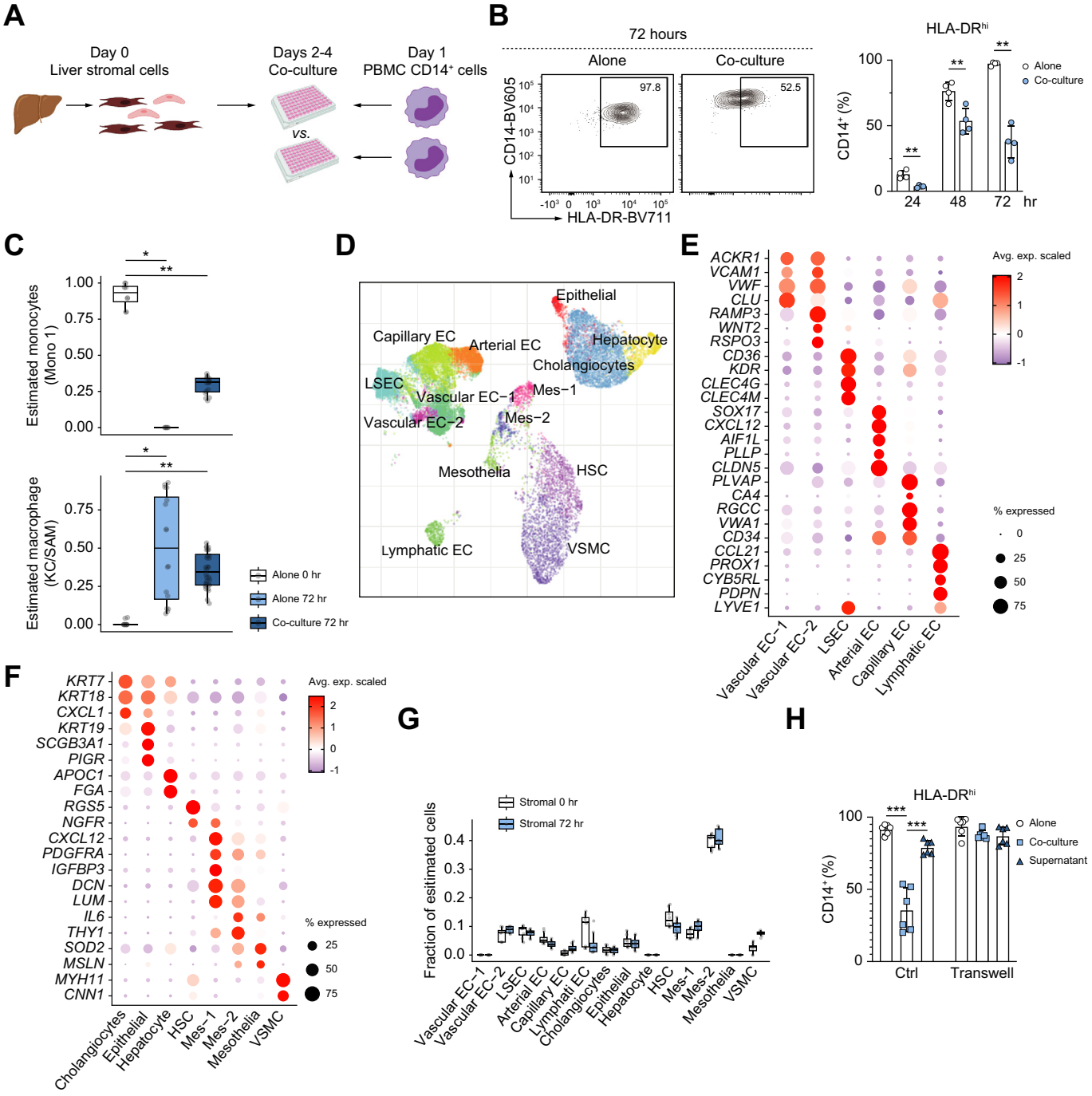

Fig. 2. Liver stromal cells restrict macrophage maturation. (A) Experimental schema. (B) Flow cytometric analysis of HLA-DR^{hi} frequency in CD14$^+$ cells cultured alone or with stromal cells. (C) DWLS boxplot showing estimated monocyte or macrophage proportions in bulk RNA-seq dataset. (D) UMAP embedding of stromal cell single-cell clusters. (E-F) Dot plots of gene expression of gene markers for (E) endothelial and (F) mesenchymal, hepatocytes, epithelial cell subsets. (G) DWLS boxplot of estimated proportions of scRNA-seq stromal sub-populations in *in vitro* bulk RNA-seq dataset. (H) Flow cytometric analysis of HLA-DR^{hi} cell frequency in CD14$^+$ cells. (B,H) Data are representative of 2 experiments with 2 PBMC donors/experiment (B) Student's *t* test; (H) One-way ANOVA Tukey's multiple comparisons; (C) Kruskal-Wallis one-way ANOVA and Dunn's multiple comparisons test; $^*p$ <0.05, $^{**}p$ <0.005, $^{***}p$ <0.0001, $^{****}p$ <0.0001. DWLS, diagonally weighted least squares; EC, endothelial cell; HSC, hepatic stellate cell; LSEC, liver sinusoidal endothelial cell; PBMC, peripheral blood mononuclear cell; RNA-seq, RNA-sequencing; scRNA-seq, single-cell RNA sequencing; UMAP, uniform manifold approximation and projection; VSMC, vascular smooth muscle cell.

Stromal cells restrict the maturation of human monocytes to macrophages

To examine how stromal cells affect macrophage maturation, we utilized a human stromal-myeloid coculture system in which primary liver stromal cells are cocultured for 0-72 hours with freshly isolated blood-CD14$^+$ cells (Fig. 2A). Stromal cells significantly reduced the transition of CD14$^+$ monocytes to macrophages by flow cytometry as evidenced by reduced frequency of HLA-DR^{hi} cells after 24 (12.7% *vs.* 3.6%), 48 (76.2% *vs.* 53.5%) and 72 (97.1 to 37.6%) hours of coculture (Fig. 2B). In agreement with this finding, bulk RNA-seq deconvolution analysis revealed that CD14$^+$ cells cocultured with stromal cells had transcriptional similarities to Mono1 and macrophage (KCs and SAMs) scRNA-seq clusters, indicating a mixture of monocytes and macrophages (Fig. 2C). Together these data indicate that primary liver stromal cells restrict the maturation of monocytes to macrophages *in vitro.*

Cultured stromal cells transcriptionally resemble a pro-fibrillar mesenchymal cell subset, Mes-2

To distinguish which subset of liver stromal cells restricts macrophage maturation *in vitro,* we compared transcriptomes of *ex vivo* liver stromal cells by scRNA-seq to *in vitro* primary liver stromal cells by bulk RNA-seq. Subcluster analysis of the stroma compartment revealed 14 non-hematopoietic cell clusters including cholangiocytes (*KRT7,KRT8*), hepatocytes (*APOC1, FGA*), mesothelia (*SOD2, MSLN*), hepatic stellate cells (*RGS5, NGFR*), vascular smooth muscle cells (*CNN1, MYH11*), 6 distinct clusters of vascular, arterial, sinusoidal, capillary, and lymphatic endothelial populations, and 2 mesenchymal sub-populations (Fig. 2D–F). We observed increased vascular endothelial cell-1, arterial and lymphatic endothelial cell populations, and decreased liver sinusoidal endothelial cell populations in cirrhotic compared to non-cirrhotic tissue (Fig. S5A).

Transcriptional alignment of scRNA-seq subsets with *in vitro* stromal cells using DWLS analysis identified mesenchymal cell subset, Mes-2, as the majority subtype and a mixture of Mes-1, hepatic stellate cells, and various endothelial populations as the minority subsets found in *in vitro* primary liver stromal cells at both 0 and 72 hours in culture (Fig. 2G). Deeper characterization of the Mes-2 stromal subset demonstrated that these cells are the primary fibrillar collagen producers, evident by increased gene expression of *COL3A1, COL1A2, COL1A1, COL5A,* and *DCN* (Fig. S5B-C). *In vitro* stromal cells express the same set of fibrillar collagen genes found in Mes-2 cells, but do not express genes found in hepatocyte and mesothelial subsets, supporting the DWLS analysis (Fig. S5D-F). These data indicate that a mixture of non-hematopoetic cells, with a dominant composition of Mes-2 mesenchymal cells limit macrophage maturation.

Stromal cells restrict macrophage maturation through a cell contact mechanism

To understand whether macrophage maturation was limited by stromal cell contact or soluble factors, we employed an *in vitro* transwell system and stromal cell supernatant transfer techniques. The *in vitro* transwell system prevents cell-cell contacts by separating stromal cells, which are plated below a 0.4 μm pore mesh filter, from CD14$^+$ cells, which are plated on top of the filter. This system allows the CD14$^+$ cells to receive signals from stromal secreted factors but restricts cell contact. Stromal cells in control plates that lack mesh filters reduced the frequency of HLA-DR^{hi} in CD14$^+$ cells (89.9% *vs.* 22.5%), but neither stromal supernatants nor stromal cells plated in transwell plates were capable of significantly reducing HLA-DR^{hi} cell frequency (Fig. 2H). Thus, stromal-myeloid cell-cell contact is the dominant mechanism by which stromal cells reduce HLA-DR^{hi} expression and restrict maturation of CD14$^+$ cells.

Stromal cells limit the differentiation of macrophages to CD9$^+$ SAMs

Because CD9$^+$ SAM subsets were increased and CD163$^+$ KC subsets decreased in cirrhotic livers by scRNA-seq analyses (Fig. 3A), we investigated if stromal cells altered myeloid cell differentiation into macrophage subsets of clinical relevance. We characterized transcriptomes of CD14$^+$ cells cultured alone or with stromal cells for 72 hours and aligned them to gene signatures of scRNA-seq myeloid cluster transcriptomes using the DWLS method. Principal component analysis demonstrated a separation of transcriptome profiles in CD14$^+$ cultured alone or with stromal cells for 72 hours (Fig. S6A). Bulk RNA-seq deconvolution analysis revealed that CD14$^+$ cells cultured alone resembled SAMs, whereas CD14$^+$ cells cocultured with stromal cells were transcriptionally similar to KCs (Fig. 3B). Gene expression analyses confirmed these similarities, as CD14$^+$ cultured alone had increased expression of *CD9, FABP5, TREM2,* like SAMs, and CD14$^+$ cells cocultured with stromal cells had increased expression of *CD163, C1QA, and CTSB,* similar to KC subsets (Fig. 3C). Further characterization of gene profiles revealed that stromal cell coculture of CD14$^+$ cells enhanced gene signatures associated with non-classical macrophage and immune effector function (*MRC1* (*CD206*), *NOD2, CCR2, FPR1, FCGR3A* (*CD16*)) (Fig. S6B-C). Likewise, the upregulated genes in stromal-cocultured CD14$^+$ cells were enriched for genes in the Gene Ontology term pathways involving immune myeloid response and effector function (Fig. S6D-E). These results indicate that stromal cells limit the differentiation of macrophages into CD9$^+$ SAMs found in cirrhosis.

Based on these findings, we hypothesized that CD9$^+$ and CD163$^+$ would be useful biomarkers for *in vitro* generated SAM-like and KC-like subsets, respectively. Flow cytometric analysis validated increased frequencies of CD163$^+$ expression in stromal cocultured CD14$^+$ cells and CD9$^+$ expression in CD14$^+$ cells cultured alone (Fig. S7A). Stromal coculture of CD14$^+$ HLA-DR^{hi} cells reduced CD9$^+$ frequency (99.2% *vs.* 42.38%) and upregulated CD163$^+$ frequency (76.5% *vs.* 95.0%) (Fig. 3D). Both conditions induced a CD163$^+$ CD9$^+$ population, indicating a degree of heterogeneity among CD14$^+$ HLA-DR^{hi} cells (Fig. S7B-C). These data support the hypothesis that stromal cell signals regulate the differentiation of macrophages and may provide protection from cirrhosis by limiting SAMs.

Soluble stromal factors are sufficient to restrict the differentiation of SAMs

Since stromal contact is required for macrophage maturation, we assessed whether contact is also required to regulate macrophage differentiation using similar *in vitro* transwell assay and stromal cell supernatant transfer techniques. Both supernatant transfer and transwell coculture methods induced KC-like CD163$^+$ and limited SAM-like CD9$^+$ expression on CD14$^+$ cells, revealing that stromal cell soluble factors are sufficient for controlling macrophage differentiation (Fig. 4A). Supernatant transfer in control plates increased the frequency of CD163$^+$

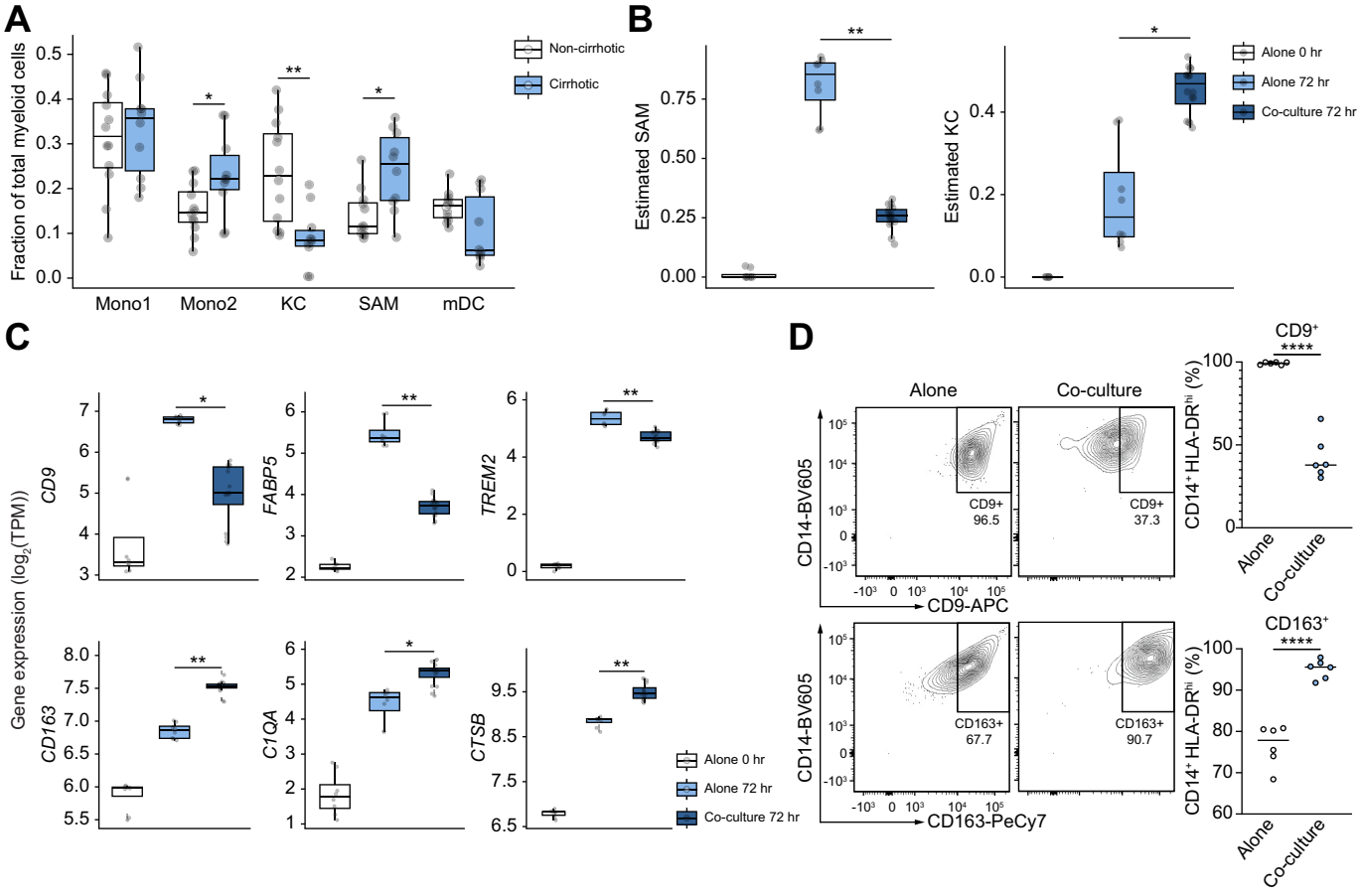

Fig. 3. Liver stromal cells reduce the differentiation of CD9⁺ SAMs. (A) scRNA-seq analysis of cell subset frequency in cirrhotic and non-cirrhotic livers. (B) DWLS boxplot of estimated SAM and KC proportion in the bulk RNA-seq data. (C) Gene expression boxplot in cultured CD14⁺cells. (D) Flow cytometric analysis of CD9⁺ and CD163⁺ frequencies in CD14⁺ HLA-DR^{hi} cells cultured for 72 hours. Data are representative of 2 experiments with 2 PBMC donors/experiment. (A-C) Wilcoxon rank sum test; (D) Student's *t* test; **p* <0.05, ***p* <0.01, ****p* < 0.001, *****p* <0.0001. DWLS, diagonally weighted least squares; KC, Kupffer cell; mDC, myeloid dendritic cell; PBMC, peripheral blood mononuclear cell; RNA-seq, RNA-sequencing; SAM(s), scar-associated macrophage(s); scRNA-seq, single-cell RNA sequencing; UMAP, uniform manifold approximation and projection.

expression (65.2% to 91.8%) and reduced the frequency of CD9⁺ expression (87.5% *vs.* 8.88%) in CD14⁺ HLA-DR^{hi} cells. Likewise, stromal cells in transwell plates increased the frequency of CD163⁺ expression (45.1% *vs.* 87.1%) and reduced the frequency of CD9⁺ expression (94.8% to 24.5%) (Fig. 4A). In order to understand whether supernatants alone can comparably regulate expression of genes indicative of myeloid cell function we analyzed the expression of M2-macrophage or non-classical monocyte markers such as CD206, CD16, and the migratory marker CCR2 on CD14⁺ cells from each experimental condition. We found comparable regulation of CD206, CD16, and CCR2 in CD14⁺ cells plated in transwell or control plates or exposed to stromal supernatant by flow cytometry (Fig. S8). These results support the finding that stromal supernatant is sufficient to alter myeloid cell function. Lastly, to assess the impact of cell survival in our system, we performed flow cytometric analysis of viable cells and found a 94% and 97% survival rate in CD14⁺ cells cultured alone or with stromal cells (Fig. S7D), ruling out a cell survival mechanism for stromal regulation of CD14⁺ cell function. These data support the concept that soluble stromal mediators are responsible for modulating the differentiation of macrophages.

The IL-6/IL-6R pathway is upregulated during stromal-myeloid interactions

We conducted annotated cognate receptor-ligand pair expression analysis on *in vitro* stromal and CD14⁺ cells using bulk RNA-seq to detect potential mechanisms controlling macrophage differentiation (Fig. 4B). We identified both soluble and contact-dependent annotated pathways but focused on soluble pathways as they are sufficient to alter differentiation. We validated a subset of identified stromal-myeloid pairs, including IL-6/IL-6R, in our *ex vivo* liver scRNA-seq data set (Fig. 4C). IL-6 protein expression was significantly increased in supernatants of CD14⁺ cells cultured with stromal cells (783,373 pg/ml) compared to CD14⁺ cells cultured alone (78.0 pg/ml), suggesting a potential role in stromal-myeloid interactions (Fig. 4D, Fig. S7E). Bulk RNA-seq analysis confirmed IL-6 expression in liver stromal cells and IL-6R expression on CD14⁺ cells (Fig. 4E). These data identify

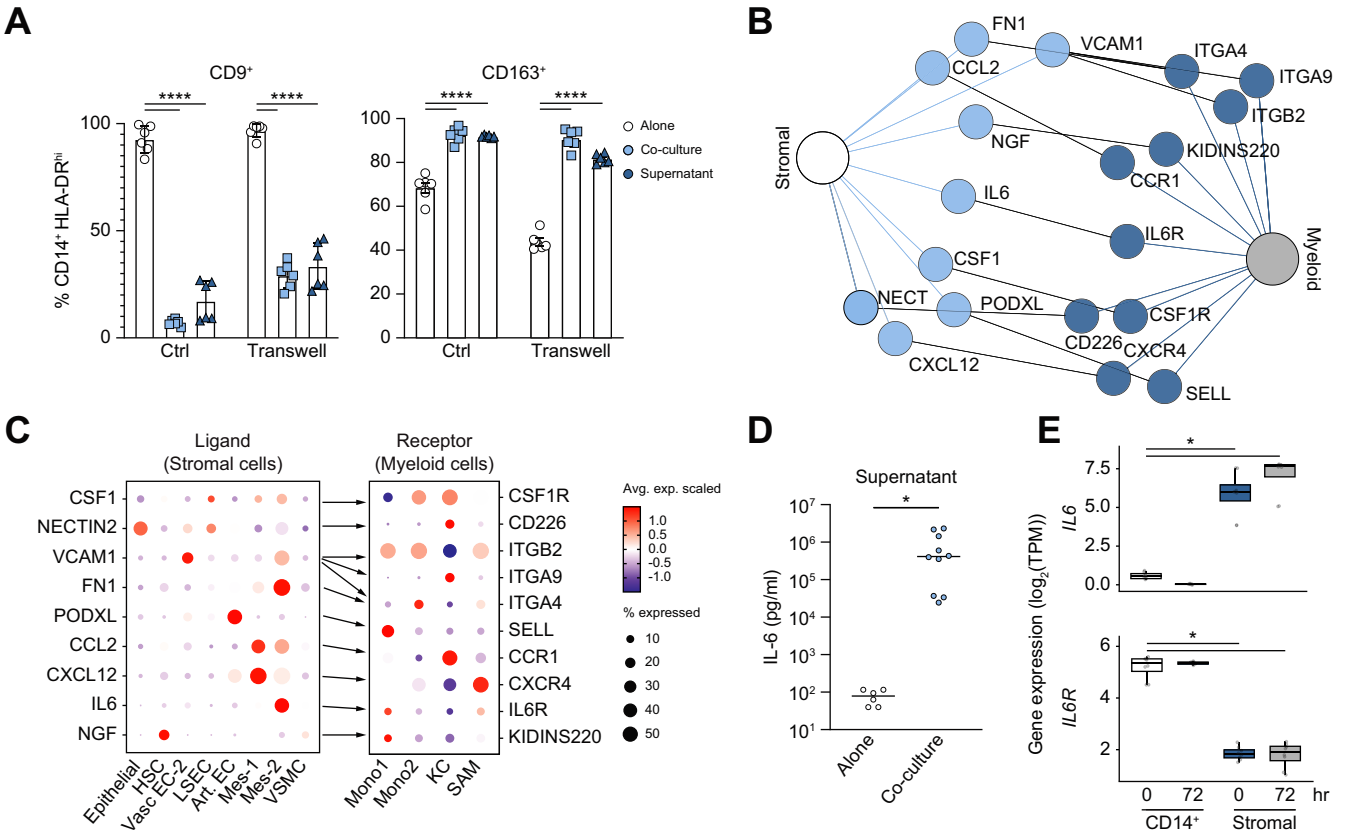

Fig. 4. Stromal soluble factors are sufficient to limit the differentiation of SAMs and the IL-6/IL-6R pathway is upregulated. (A) Flow cytometric analysis of CD9$^+$ and CD163$^+$ frequencies in HLA-DR^{hi} cells for 72 hours. Representative of 2 experiments with 2 PBMC donors/experiment. (B) Candidate ligand-receptor pairs from *in vitro* bulk RNA-seq dataset. (C) Bubble heatmap of ligand-receptor pair expression in stromal and myeloid scRNA-seq subsets. (D) IL-6 protein expression in supernatants of CD14$^+$ cells cultured alone or with stromal cells for 72 hours. (E) Bulk RNA-seq analysis of IL-6 and IL-6R gene expression in cultured cells. (A) One-way ANOVA Tukey's multiple comparisons; (D) Welsh's T test; (E) Wilcoxon rank sum test; $^*p$ <0.05, $^{**}p$ <0.01, $^{***}p$ < 0.001, $^{****}p$ <0.0001. EC, endothelial cell; HSC, hepatic stellate cell; LSEC, liver sinusoidal endothelial cell; PBMC, peripheral blood mononuclear cell; RNA-seq, RNA-sequencing; scRNA-seq, single-cell RNA sequencing; UMAP, uniform manifold approximation and projection; VSMC, vascular smooth muscle cell.

stromal-myeloid IL-6-IL-6R interactions as a plausible mechanism for controlling macrophage differentiation.

Stromal-derived soluble IL-6 is sufficient to alter macrophage differentiation

To investigate whether stromal IL-6 impacts macrophage subset differentiation, we blocked IL-6 signaling in cocultures and supernatant transfer experiments using anti-IL6R antibody. Anti-IL-6R treatment reduced the ability of stromal cell cocultures and supernatants to limit CD9$^+$ SAM-like cells and induce CD163$^+$ KC-like cells (Fig. 5A-B). Anti-IL6R treatment in supernatant transfer and cocultured systems resulted in a frequency of CD9$^+$ cells of 72.5% and 67.5% compared to 45.6% and 43.3% in isotype controls, respectively. Likewise, anti-IL-6R treatment in supernatant transfer and cocultured systems resulted in a CD163$^+$ cell frequency of 43.6% and 14.9% compared to 87.5% and 85.3% in isotype controls, respectively. We investigated how robust the ability of anti-IL6R blockade to dampen stromal cell regulation of macrophage differentiation was across multiple CD14$^+$ (n = 3) donors and primary liver stromal cells (n = 2) by analyzing CD9$^+$ and CD163$^+$ cell frequencies (Fig. S7F). We found similar frequencies of CD9$^+$ and CD163$^+$ cells in each donor suggesting that the role for IL-6 is not donor specific. Together, these studies identify secreted IL-6 as a mechanism for stromal cell restriction of SAM differentiation.

Previous studies have demonstrated that signaling through membrane bound IL-6R via classic IL-6 signaling can be anti-inflammatory and facilitate the induction of the hepatic acute phase response.[19] Therefore, understanding the signaling mechanism by which IL-6 controls macrophage differentiation may have therapeutic implications. Therefore, we asked whether classic IL-6 or trans IL-6 signaling, via IL-6-IL-6R complexes, could modulate CD14$^+$ macrophage differentiation. Classic IL-6 signaling involves the interaction of IL-6 with membrane bound IL-6R and gp130 protein, whereas trans IL-6 signaling allows IL-6 to complex with soluble IL-6R and interact with gp130 protein on the cell membrane.[19] We assessed CD9$^+$ and CD163$^+$ expression on CD14$^+$ cells after culture for 72 hours alone, with recombinant IL-6 (rIL-6) (classic), or with IL-6/IL-6R fusion (FC-IL-6) (trans) protein (Fig. 5C). Both rIL-6 and FC-IL-6 protein treatments mimicked stromal cell downregulation of CD9$^+$ and upregulation of CD163$^+$ expression. rIL-6 and FC-IL-6 treatment downregulated the frequency of CD9$^+$ cells (79.1% to 44% and 47.1%) and upregulated the frequency of CD163$^+$ cells (72.8% *vs.* 85.3% and 86.9%). These data suggest that classic and trans IL-6 signaling are both capable of modulating macrophage differentiation.

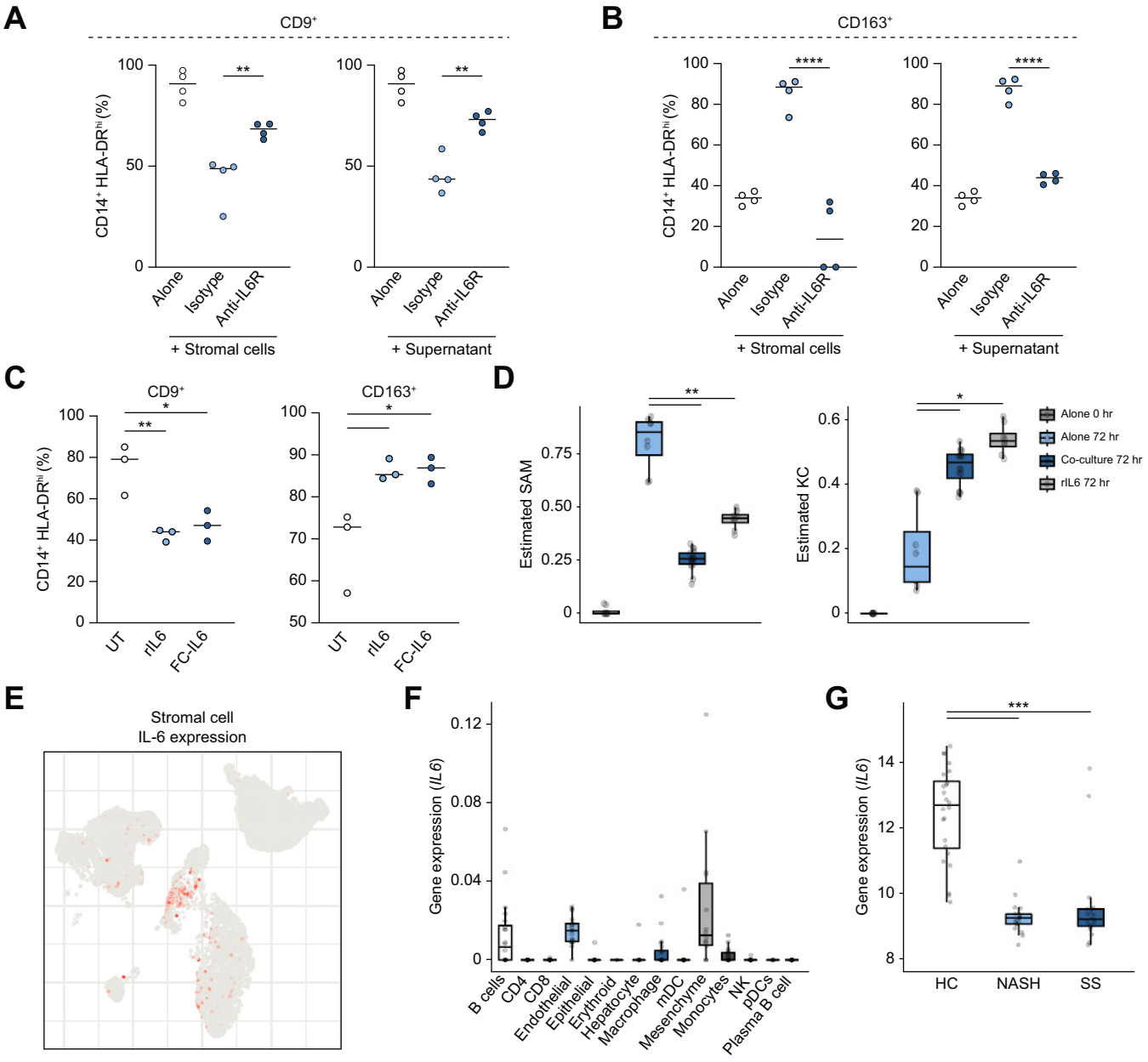

Fig. 5. Soluble IL-6 from stromal cells restricts CD9⁺ SAM differentiation. (A-B) Flow cytometric analysis of (A) CD9⁺ and (B) CD163⁺ frequency in CD14⁺ HLA-DR^{hi} cells following antibody treatment for 72 hours. (C) Flow cytometric analysis of CD9⁺ and CD163⁺ frequency in CD14⁺ cells cultured alone, with rIL-6, or FC-IL-6 for 72 hours. (D) DWLS boxplot showing estimated SAM and KC cell proportions from bulk RNA-seq data after rIL-6 treatment. (E-F) scRNA-seq analysis of (E) IL-6 expression in stromal cells and (F) in cell subsets. (G) Expression of IL-6 in whole tissue microarray from healthy liver (HC), NASH, and SS. (A-C) Data are representative of 2 experiments with n = 2 PBMC donors. One-way ANOVA Tukey's multiple comparisons; (D,G) Kruskal-Wallis one-way ANOVA and Dunn's multiple comparisons test; *p <0.05, **p <0.05, ***p <0.001, ****p <0.0001. DWLS, diagonally weighted least squares; FC-IL-6, fusion IL-6; KC, Kupffer cell; mDC, myeloid dendritic cell; NASH, non-alcoholic steatohepatitis; NK, natural killer; PBMC, peripheral blood mononuclear cell; pDC, plasmacytoid dendritic cell; rIL-6, recombinant IL-6; RNA-seq, RNA-sequencing; SAM, scar-associated macrophage; scRNA-seq, single-cell RNA sequencing; SS, simple steatosis; UMAP, uniform manifold approximation and projection.

DWLS analysis confirmed that rIL-6-treated CD14⁺ cells transcriptionally aligned most closely to KC liver subsets, similar to stromal coculture-treated cells (Fig. 5D). However, anti-IL6R blockade, rIL-6, nor FC-IL-6 did not have a strong effect on the maturation of monocytes to macrophages (Fig. S9A-B). These data further support the hypothesis that IL-6 signaling skews macrophage differentiation away from SAM-like macrophages and towards KC-like macrophages but does not impact macrophage maturation. These findings highlight an important role for IL-6 signaling in the regulation of macrophage differentiation and suggest that stromal IL-6 may play a protective role in inhibiting or slowing the development of liver disease by maintaining KC-like macrophage subsets in liver tissue.

IL-6 is produced by stromal cells in human livers

We investigated which cell subsets produce IL-6 in liver tissue using our scRNA-seq datasets to further relate our *in vitro* findings to human liver disease. We asked where IL-6 is produced in

stromal cell subsets and more broadly throughout liver tissue (Fig. 5E-F). Like in our *in vitro* coculture system, stromal IL-6 is primarily produced by mesenchymal liver cells, Mes2, with a lesser proportion of IL-6 being produced by endothelial cells and B cells. These observations emphasize the importance of Mes-2 cells in local production of IL-6.

Local IL-6 expression is reduced in the livers of patients with early-stage liver disease

Our data suggest that IL-6 may be protective against the development of cirrhosis by skewing away from SAMs, yet IL-6 is increased in the blood of patients with cirrhosis, correlating its systemic expression to liver pathology.[20–22] Therefore, we investigated whether local IL-6 expression was altered during liver disease. We examined liver IL-6 gene expression during various stages of liver disease using both our scRNA-seq data and publicly available data sets.[23] First, we compared IL-6 expression in liver mesenchymal cells and endothelial cells during late-stage liver disease using cirrhotic and non-cirrhotic samples (Fig. S10A). IL-6 expression was enhanced in 1 cirrhotic donor, but we found no significant difference between cirrhotic and non-cirrhotic samples from the remaining 11 donors (Fig. S10B-C). We also examined cirrhotic and non-cirrhotic samples by immunohistochemistry and observed similar clustering of IL-6 expression in hepatocytes randomly distributed in both non-cirrhotic and cirrhotic tissue (Fig. S10D). We next asked whether local IL-6 expression was changed during early-stage disease by analyzing expression in bulk liver tissue using a publicly available microarray dataset that includes 63 liver samples: 24 healthy livers, 20 simple steatosis and 19 non-alcoholic steatohepatitis (NASH).[23] IL-6 expression was significantly decreased in livers with steatosis or NASH compared to healthy livers (Fig. 5G). Although additional studies are needed to clarify the contribution of local and systemic IL-6 during various stages of liver disease, our data suggest that local IL-6 expression is reduced during the early stages of disease, which may permit the differentiation of SAMs and perpetuate damage leading to cirrhosis.

Since IL-6 plays multi-faceted roles in many inflammatory diseases, we further aimed to identify potential pathways of additional or combinatorial importance to IL-6/IL-6R signaling. We performed a comprehensive receptor-ligand pair analysis for *ex vivo* and cultured stromal-myeloid cells (Fig. S11A-B). We limited our analysis to receptor-ligand pairs involved in pathways identified in both bulk RNA-seq and scRNA-seq datasets in order to detect pairs with the highest potential to translate from the coculture system to biological processes occurring in liver tissue. This analysis revealed increased ligand CSF1 in cultured stromal cells and Mes-2 cells and the receptor CSF1R in cultured $CD14^+$ cells and KC cells, but not SAMs, indicating that the CSF1-CSF1R pathway may also play a role in facilitating stromal-dependent macrophage differentiation.

Discussion

Cirrhosis is the most common cause of liver-related mortality across the globe and its prevalence is increasing annually.[1,24] Despite the worldwide burden of cirrhosis, treatment options to effectively target late-stage liver disease are lacking.

We have discovered a novel role for liver stromal cells in regulating the maturation and differentiation of macrophages. We find that liver stromal cells limit monocyte maturation into macrophages and that stromal IL-6 production restricts myeloid cell differentiation into SAM-like $CD9^+$ macrophages. To determine how stromal cells impact myeloid cell functionality and investigate signals that control these interactions, we generated a biobank of human primary liver stromal cell lines that were cocultured with myeloid cells. We translated observations from the stromal-myeloid coculture system to human liver disease by aligning the transcriptomics of cocultured stromal and $CD14^+$ cells to single stromal and myeloid cells from non-cirrhotic and cirrhotic tissue using bulk RNA-seq and scRNA-seq techniques, respectively. We determined that liver stromal cells that transcriptionally resemble the stromal cell subset, Mes-2, can limit the maturation and alter the differentiation of macrophages *in vitro*. We find that both Mes-2 and *in vitro* stromal cells have increased expression of fibrillar collagen-producing genes, revealing an activated stromal phenotype. Increased collagen production is observed during wound healing, a process that requires M2 macrophages. We find that $CD14^+$ cells cultured with liver stromal cells transcriptionally resemble non-cirrhotic $CD163^+$ KCs, whereas those cultured alone resemble cirrhotic $CD9^+$ SAMs. Deeper analysis of gene profiles revealed that $CD163^+$ $CD14^+$ cells express non-classical or M2-macrophage markers such as $CD16^+$ and $CD206^+$ and migratory marker $CCR2^+$, indicating that stromal cells may induce macrophage phenotypes important for wound healing. Together, these data support that *in vitro* stromal and cocultured myeloid cells may be transcriptionally similar to cells found during tissue repair in healthy liver tissue.

We identified stromal cell soluble IL-6 as a mechanism by which stromal cells support the differentiation of $CD163^+$ cells and limit $CD9^+$ cells. Contrary to systemic IL-6 levels, which increase during cirrhosis, we found no difference in local IL-6 levels in healthy and cirrhotic liver tissue but observed decreased levels in early-stage liver tissue compared to healthy controls. Our results imply that local stromal-derived IL-6 in healthy liver tissue may inhibit early-stage liver disease by limiting the differentiation of $CD9^+$ SAMs and aid in effective wound healing processes. Likewise, comparable levels of IL-6 seen in cirrhotic and non-cirrhotic tissue may reflect an unsuccessful attempt of cirrhotic stromal cells to decrease the frequency of SAMs present in severe disease. Our findings should prompt future research into how local and systemic IL-6 signaling may vary during early and late phases of liver disease and how IL-6 regulates myeloid cell function to control the progression of disease.

It is also possible that IL-6 is sufficient, but not exclusive, in facilitating stromal-mediated myeloid cell differentiation towards $CD163^+$ KCs. CSF1/CSF1R, NOTCH, and BMP signaling have been demonstrated to regulate murine macrophage differentiation.[12] We identified stromal expression of *CSF1* in cultured and Mes-2 cells and expression of the receptor, *CSF1R*, in cultured and Mono1/KC subsets in liver tissue, warranting further investigation into the role of CSF1/CSF1R signaling in stromal-myeloid interactions during liver disease. These findings encourage future studies into additional signaling pathways that may be leveraged to work synergistically with IL-6 to protect against disease.

Lastly, we determined that cultured HLA-DR^{hi} $CD14^+$ cells most closely resemble macrophages in both human liver and tumor tissue, identifying HLA-DR^{hi} as a valuable marker to distinguish human monocytes from macrophages in *in vitro* and *ex vivo* studies. We demonstrated that freshly isolated peripheral

$CD14^+$ monocytes will mature into macrophages after 3 days in culture, providing much needed insight into the characteristics and classification of cultured human PBMC-derived $CD14^+$ cells commonly used in *in vitro* systems.

A limitation of our study is that the transcriptional alignment of *in vitro* to *ex vivo* cells is confined to cells detected in scRNA-seq analysis. Cell types sensitive to death during processing, such as hepatocytes, have a relatively lower abundance in scRNA-seq datasets compared to their frequency in liver tissue. Additionally, hepatocytes are challenging to culture and passage *in vitro*, resulting in their absence in our coculture system. The role of hepatocytes in the development of liver disease is intriguing due to their observed ability to produce IL-6 in liver tissue by immunohistochemical analysis. While these technical limitations do not detract from the conclusions made regarding stromal-myeloid interactions, further work is needed to address the role of hepatocytes in the regulation of human myeloid cell maturation and differentiation.

In summary, our studies begin to unravel the complexity of stromal-myeloid relationships and identify potential pathways that may be clinically relevant for treating fibrosing liver disease. More broadly, our studies provide a framework for investigating the crosstalk between stromal cells and macrophages during tissue homeostasis, injury, and disease. Our stromal-myeloid *in vitro* platform can be broadly applied to investigate mechanisms by which stromal cells modulate macrophage maturation and differentiation. Our findings raise the question of whether stromal cells are also capable of restricting the maturation and differentiation of macrophages *in vivo* during human liver disease and other inflammatory pathologies. A deeper understanding of stromal-myeloid interactions across a spectrum of liver disease has the potential to identify novel therapeutic targets for treating chronic fibrotic liver diseases.

Abbreviations

DWLS, diagonally weighted least squares; FC-IL-6, fusion IL-6; HSC, hepatic stellate cell; KC, Kupffer cell; NASH, non-alcoholic steatohepatitis; PBMC, peripheral blood mononuclear cell; rIL-6, recombinant IL-6; RNA-seq, RNA sequencing; SAM, scar-associated macrophage; scRNA-seq, single-cell RNA sequencing.

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Conflicts of interest

AS has patents/pending royalties on the PD-1 pathway from Roche and Novartis. AS is on advisory boards for Surface Oncology, SQZ Biotechnologies, Elpiscience, Selecta, Bicara and Monopteros. AS has received research funding from Novartis, Roche, UCB, Ipsen, Quark, Merck, AbbVie and Moderna. PK serves on the Scientific Advisory Board to Celsius Therapeutics Inc and Biomage Inc.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

EB, SM, IL, SG, MP, MN performed experiments and analyzed data. JM and LZ scored pathology of tissue. FS helped establish methods. CL, SX, and LZ conducted IHC staining and analysis. AS, EB, PK, SM conceptualized project and supervised experiments and data analysis. All authors discussed results and helped prepare this manuscript.

Data availability statement

Single-cell RNA sequencing and bulk RNA sequencing data have been deposited in Gene Expression Omnibus (GEO) database under GEO: GSE168933.

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Supplementary data

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Author names in bold designate shared co-first authorship

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